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Age of Inoculum Strongly Influences Persister Frequency and Can Mask Effects of Mutations Implicated in Altered Persistence▽†

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The majority of cells transferred from stationary-phase culture into fresh medium resume growth quickly, while a few remain in a nongrowing state for longer. These temporarily nonproliferating bacteria are tolerant of several bactericidal antibiotics and constitute a main source of persisters. Several genes have been shown to influence the frequency of persisters in *Escherichia coli*, although the exact mechanism underlying persister formation is unknown. This study demonstrates that the frequency of persisters is highly dependent on the age of the inoculum and the medium in which it has been grown. The *hipA7* mutant had 1,000 times more persisters than the wild type when inocula were sampled from younger stationary-phase cultures. When started after a long stationary phase, the two displayed equal and elevated persister frequencies. The lower persister frequencies of *glpD*, *dnaJ*, and *surA* knockout strains were increased to the level of the wild type when inocula aged. The *mqsR* and *phoU* deletions showed decreased persister levels only when the inocula were from aged cultures, while *sucB* and *ygfA* deletions had decreased persister levels irrespective of the age of the inocula. A dependency on culture conditions underlines the notion that during screening for mutants with altered persister frequencies, the exact experimental details are of great importance. Unlike ampicillin and norfloxacin, which always leave a fraction of bacteria alive, amikacin killed all cells in the growth resumption experiment. It was concluded that the frequency of persisters depends on the conditions of inoculum cultivation, particularly its age, and the choice of antibiotic.

Genetically homogeneous bacterial cultures can give rise to subpopulations with different physiological properties (38). This kind of heterogeneity can be demonstrated in terms of growth resumption when stationary-phase bacteria are diluted in fresh medium: some cells start growth immediately, some later, and some do not recover during the period of observation (2, 15, 33). Variation in recovery could reflect the random nature of cell damage (8). Alternatively, as rapidly recovering bacteria are more vulnerable to harmful environmental conditions than dormant ones, the wide range of growth resumption times could represent an ecological strategy (11, 21, 39). For example, the majority of bactericidal antibiotics kill growing bacteria, and nongrowing cells survive (41). Furthermore, antibiotic-sensitive growing cultures cannot be sterilized by bactericidal drugs, suggesting the existence of a small subpopulation of nongrowing bacteria (4). These bacteria, called persisters, can survive antibiotic treatment and resume growth after removal of the drug (23).

Several mutations lead to increased or decreased persister levels (9, 10, 13, 19, 24, 25, 27, 40). A classical mutant with increased persister frequency is the *hipA7* strain (27), which carries mutations in the gene coding for the toxin in the HipAB toxin-antitoxin pair (21). This mutation decreases the affinity of the toxin for antitoxin, leading to increased toxin activity (34). The product of *hipA* is a kinase that phosphorylates EF-Tu, although the exact mechanism underlying the formation of HipA-dependent persisters remains unclear (36). In addition to manipulating the toxin-antitoxin systems, there are other ways of changing the persister frequency, for example, mutating the genes involved in metabolism, like *sucB* (25), *phoU* (24), and *glpD* (40), or overexpressing toxic proteins (42).

It has been demonstrated that the majority of persisters in batch culture experiments come from the stationary-phase inoculum (15, 17). These bacteria do not divide after dilution in fresh medium and throughout antibiotic treatment but resume growth and form colonies when plated. Aging of cultures delays regrowth after reinoculation and on agar plates (22, 32), suggesting a strong effect on persister formation. This study investigated how the age of the stationary-phase inoculum and the medium in which it is grown influence the persister formation of wild-type (wt) *Escherichia coli* and a range of previously described mutants with altered persister frequencies, including the *hipA7* strain.

MATERIALS AND METHODS

Bacterial strains and plasmids. MG21 (wild type) and MG22 (*hipA7*) were derived from K-12 MG1655 [F$^-$ λ$^-$ *ilvG rfb-50 rph-1*] (5) by P1 transduction from HM21 and HM22 (27), respectively, using selection on tetracycline. The presence of the correct *hipA* allele was verified using sequencing. The MG21Δ*hipBA* strain was constructed from MG21 according to the method of Datsenko and Wanner (7). Strains MG1655, MG21, MG22, and MG21Δ*hipBA* were transformed with the plasmid pETgfp-mut2-AGGAGG(3) carrying a green fluorescent protein (GFP) *mut2* gene under the control of an IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible promoter and a kanamycin resistance marker (43). Single-knockout (Δ*glpD*, Δ*sucB*, Δ*mqsR*, Δ*recA*, Δ*dnaJ*, Δ*surA*, Δ*ygfA*, Δ*phoU*, Δ*acnB*, and Δ*mdh*) strains were from the Keio collection (1). The antibiotic resistance cassette was removed in the Δ*icdA* and Δ*mdh* strains. The isogenic parental strain was K-12 BW25113 [Δ(*araD-araB*)*567* Δ*lacZ4787*(::*rrnB-3*) lambda *rph-1* Δ(*rhaD-rhaB*)*568 hsdR514*] (7). Some knockout strains (Δ*glpD*, Δ*mqsR*, Δ*dnaJ*, Δ*surA*, Δ*ygfA*, Δ*phoU*, and Δ*icdA* strains) were also constructed in a clean background, using the primers published for making

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the Keio collection (1), by the method of Datsenko and Wanner, including antibiotic resistance cassette removal (7). Where indicated, the BW25113 and Δ*sucB* strains were transformed with the plasmid pETgfp-mut2-AGGAGG(3)-CAT, which was derived from pETgfp-mut2-AGGAGG(3) (43) by removing the kanamycin resistance marker with PagI and replacing it with a chloramphenicol resistance marker from pBAD33 cut out with NheI and Bsp119I. This plasmid was used in experiments containing kanamycin-resistant strains. The SucB coding region was amplified from the wt *E. coli* (BW25113) genome by PCR and cloned into the pBAD33 expression vector under the control of an arabinose-inducible promoter. The resulting pBAD-sucB plasmid was used to transform a Δ*sucB* strain from the Keio collection (the Kan^r cassette was removed from the chromosome). The persister formation test for this strain was carried out in the presence of 1 mM arabinose to induce SucB expression.

Growth media and growth conditions. To ensure the reproducibility of results, Lennox LB medium (Difco Laboratories) was prepared by filter sterilization rather than autoclaving. MOPS (morpholinepropanesulfonic acid) medium (28) was supplemented with 0.1% glucose (MOPS Glc) or 0.2% glycerol (MOPS Gly). MOPS medium with a decreased concentration of ammonia (2.7 mM rather than 9.52 mM; MOPS − N) or a decreased concentration of phosphate (0.132 mM rather than 1.32 mM; MOPS − P) or with amino acids (20 various L-amino acids at a concentration of 100 μg/ml each; MOPS AA) was supplemented with 0.1% glucose. LB plates containing 1.5% agar were used. Media for strains with the plasmid pETgfp-mut2-AGGAGG(3) contained kanamycin (25 μg/ml; Amresco), and those for strains with the plasmids pETgfp-mut2-AGGAGG(3)-CAT and pBAD-sucB contained chloramphenicol (25 μg/ml; AppliChem). For green fluorescent protein (GFP) induction, IPTG (1 mM) was added when indicated. Bacteria were grown at 37°C. Liquid cultures were grown aerobically on a shaker.

Preparation of DMSO stocks. Overnight cultures were diluted 1:100 in fresh medium and grown aerobically to the exponential phase. At an optical density (A_{600}) of 0.15 to 0.2 (MOPS Glc) or 0.8 (LB), dimethyl sulfoxide (DMSO) was added to a final concentration of 8%, and the culture was immediately frozen in 120-μl aliquots at −80°C.

Persister formation test. Cultures were started by diluting (1:20 to 1:200) DMSO stocks of the respective strains in fresh medium (LB or MOPS Glc) supplemented with IPTG when indicated. For inoculating LB medium, the stocks prepared in LB were used, and the MOPS Glc stocks were used for MOPS Glc. Cultures were grown aerobically to the stationary phase. The beginning of the stationary phase was defined as the time when the optical density of the culture did not increase >5% for 30 min. At the indicated times, cultures were diluted 1:100 in fresh medium (LB or MOPS Glc) containing ampicillin (200 μg/ml; Actavis), norfloxacin (5 μg/ml; Sigma), or amikacin (25 μg/ml; Sigma) and lacking IPTG. The persister levels of the diluted cultures were determined after 3, 9, or 24 h of incubation in the presence of antibiotic. Samples were diluted in sterile 1× phosphate-buffered saline (PBS) and plated on LB agar plates, which were incubated overnight, and colonies were counted the next day. Immediately after the dilution of stationary-phase cultures, the total culturable cell count was determined by plating.

Length of the stationary phase and growth resumption. Stationary-phase cultures were prepared and diluted in fresh medium (supplemented with IPTG) as described above using DMSO stocks of the respective strains transformed with a plasmid for GFP induction. At the indicated times, the cultures were diluted 1:100 in fresh ampicillin-containing (200 μg/ml; Actavis) medium (LB or MOPS Glc) lacking IPTG. The diluted cultures were aerobically incubated on a shaker, and growth resumption was followed over a 24-h period by taking samples and analyzing them using flow cytometry. The samples were prepared by mixing 50 μl of culture with an equal volume of 30% glycerol in 1× PBS and frozen at −80°C pending analysis by flow cytometry (LSR II; BD Biosystems). Nondividing cells were distinguished by high GFP levels. The software packages FACSDiva and FlowJo were used to visualize the data.

Effect of the stationary-phase medium on growth resumption. Cultures were started by diluting (1:20) MG1655 MOPS Glc DMSO stocks into various fresh media supplemented with IPTG. At the times indicated in Fig. S1 in the supplemental material, the cultures were diluted 1:100 in fresh LB lacking IPTG. The diluted cultures were further incubated, and samples were taken after 4 h. The samples were prepared by mixing 50 μl of culture with an equal volume of 30% glycerol in 1× PBS and frozen at −80°C until flow cytometry analysis was carried out (LSR II; BD Biosystems). Nondividing cells were distinguished by high GFP levels. The software packages FACSDiva and FlowJo were used to visualize the data.

MIC of ampicillin. The MIC test was performed according to the broth microdilution method (45) with the exception that LB medium was used.

RESULTS

The *hipA7* strain causes high persistence compared with the wild type only when the inoculum is from the early stationary phase. The *hipA7* strain (27) has been used in numerous studies and is reported to form several orders of magnitude more persisters than the wild type. Persister frequencies for the wild-type and *hipA7* strains were determined using inocula of different ages. Stationary-phase cultures were diluted in ampicillin-containing media and incubated for 3 h; cells that resumed growth during this period were lysed with ampicillin. The number of live cells was determined by plating and counting the colonies after overnight incubation. The number of unlysed, nonproliferating bacteria was determined from the same ampicillin-containing cultures by using flow cytometry (15, 33).

As expected, the *hipA7* mutant grown in LB demonstrated 1,000-fold-higher persister frequencies when the inocula were taken from younger stationary-phase cultures (Fig. 1A, triangles). However, this difference diminished and finally disappeared at later time points, as the frequency of persisters in the wild-type strain increased substantially and reached the level of the *hipA7* mutant after 18 h in the stationary phase. As the inocula aged, the frequency of persisters increased in the *hipA7* mutant, but more slowly than in the wild type.

This tendency held true when MOPS Glc medium (28) was used in place of LB (Fig. 1B, triangles), although the time span for changes in the minimal medium was longer (days) than for LB (hours). Unexpectedly, persister levels for *hipA7* inoculum grown in MOPS Glc were higher at the first stationary-phase time point than at the next time point.

The strain- and inoculum age-specific differences in persister levels were not dependent on the time of the ampicillin treatment. The differences observed after 3-h treatments were maintained after 9-h and 24-h treatments. However, the absolute level of persisters decreased slowly (see Fig. 3A). The trend continued if the cultures inoculated with 18-h stationary-phase cells were incubated with ampicillin for extended periods (48 h or 72 h), showing a general decrease in persister levels but no differences between the wild-type and *hipA7* strains (data not shown). Similar behavior was observed without GFP induction (see Fig. S1 in the supplemental material), indicating that overexpression of the marker protein did not influence the results. The *hipBA* deletion strain did not differ from the wild-type strain in its ability to form persisters (Fig. 1).

The number of cultivable cells decreased slowly and at the same rate in the three strains tested (Fig. 1, squares). There was no difference in viability between the wild type and the *hipAB* deletion strain, as previously described (16). However, this study used different media, cultivation conditions, *E. coli* strain background, and viability detection methods than previous studies, so the results are not directly comparable. The increase in the number of cells that became uncultivable and did not start growth after plating may be one explanation as to why the persister level started to decrease slowly after 18 h of stationary phase in LB.

The number of inoculated cells that did not lyse during the 3-h ampicillin treatment increased steadily throughout the stationary phase until it exceeded the CFU count (Fig. 1, circles).

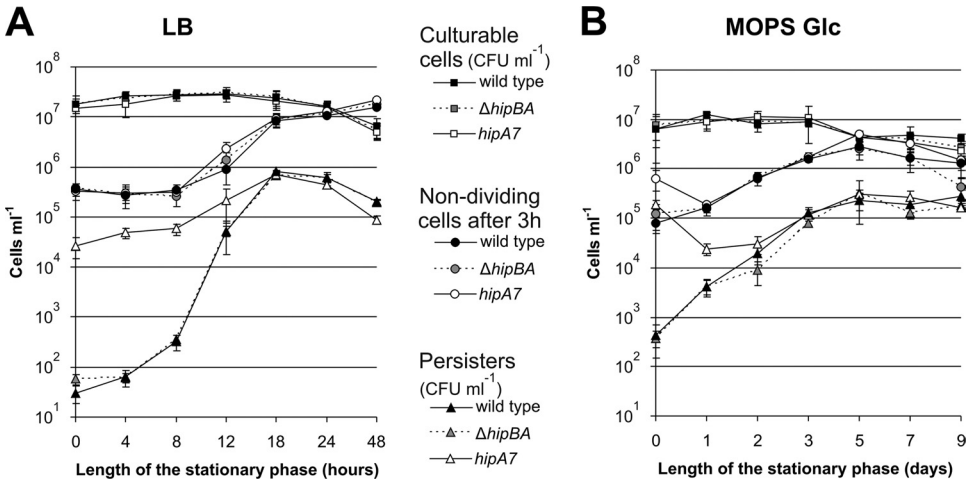

FIG. 1. Dependence of CFU, nondividing cells, and persisters on the length of the stationary phase. Stationary-phase cells of strains MG21 (wild type), MG22 (*hipA7*), and MG21Δ*hipBA* grown in LB (A) or MOPS Glc (B) and containing GFP were diluted in ampicillin-containing fresh medium (LB or MOPS, respectively) at the indicated times during the stationary phase. The beginning of the stationary phase was defined as the time when the optical density of the cultures did not increase >5% for 30 min. The number of CFU was determined by plating samples on LB plates. The number of nondividing cells was measured using flow cytometry 3 h after dilution. The persister frequency of the diluted cultures was determined by plating samples on LB 3 h after dilution and ampicillin treatment. The values represent the means of at least three independent experiments. The error bars indicate the standard errors.

Throughout the stationary phase, the unlysed-cell counts were comparable at each time point in the three strains tested. It is important to note that persisters formed in only <20% of the cells that were unlysed during plating. For the LB-grown wild-type strain, this ratio was <1:1,000 when the inocula were taken at the beginning of the stationary phase (Fig. 1A). This demonstrates that the majority of cells refractory to ampicillin lysis are not persisters, contrary to previous beliefs (18).

An increase in persister levels surviving the 3-h ampicillin treatment should manifest itself in terms of a delay in growth resumption after dilution in fresh medium. This is indeed the case (Fig. 2); the longer the stationary phase, the longer the cells take to resume growth. In LB, this delay in growth resumption was followed by a rapid loss of cultivability, resulting in the majority of cells remaining in a nondividing state as long as 24 h after dilution (Fig. 2A). In MOPS Glc, the bacteria retained the ability to resume growth longer (Fig. 2B).

The growth resumption of cells grown in a wider variety of MOPS-based media (28) supplemented with amino acids and containing a reduced amount of nitrogen or phosphorus or having the glucose replaced by glycerol as the carbon source was investigated. The nondividing cells were counted 4 h after dilution in fresh LB. The differences between MOPS Glc and other defined media were insignificant (see Fig. S2 in the supplemental material). Therefore, MOPS Glc medium was used throughout the study.

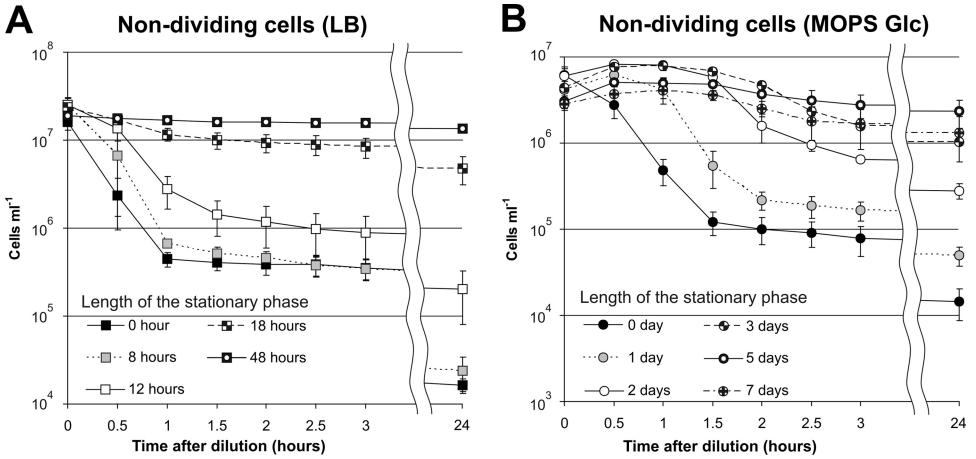

FIG. 2. Changes in growth resumption dynamics. Stationary-phase cells of strain MG21 (wild type) grown in LB (A) or MOPS Glc (B) containing GFP were diluted in fresh medium (LB and MOPS Glc, respectively) at the indicated times during the stationary phase. The beginning of the stationary phase was defined as the time when the optical density of the cultures did not increase >5% for 30 min. The number of nondividing cells was measured using flow cytometry by counting cells retaining high GFP content. The values represent the means of at least three independent experiments. The error bars indicate the standard errors.

It is important to note that LB medium was sterilized by filtration. Autoclaving LB medium led to fluctuations in quantitative results; the numbers of persisters and nondividing cells at particular time points were difficult to reproduce. Several chemical conversions occur during the autoclaving of growth media (30, 44), leading to changes in the growth-supporting properties of those media (11, 31). The chemical effect of autoclaving depends on several factors, including the volume of material and type of autoclave (6, 44), and this can decrease the reproducibility of results. These effects could be particularly relevant in very complex media, such as LB (29, 37).

Amikacin kills *E. coli* irrespective of inoculum age. The *hipA7* mutant was selected by repeated rounds of treatment with ampicillin, a cell wall synthesis inhibitor (27). This mutant has been demonstrated to have an increased tolerance for nalidixic acid (quinolone) but does not significantly influence the bactericidal effect of kanamycin (aminoglycoside) (35). This suggested that persisters might not be universally tolerant of all antibiotics. Therefore, the wild-type and *hipA7* strains of *E. coli* were subjected to treatment with norfloxacin (fluoroquinolone) and amikacin (aminoglycoside) to investigate whether the age of the inoculum affected the ability of these antibiotics to kill bacteria.

The results from the norfloxacin treatment (Fig. 3B) were comparable to those obtained using ampicillin (Fig. 3A). When wild-type cells were transferred to fresh medium after 4 h in the stationary phase, considerably fewer norfloxacin-refractory persisters were produced than in 18-h and 36-h inocula (Fig. 3B). Inoculated cells of the *hipA7* mutant generated elevated persister levels after 4 h in the stationary phase, and after a longer time in the stationary phase, they displayed the same number of norfloxacin-refractory cells as the wild type. These trends were maintained regardless of the duration of antibiotic treatment.

Incubation with amikacin led to rapid elimination of all cultivable cells, regardless of the age of the inoculum or the strain background (Fig. 3C). After 3 h of treatment, amikacin had effectively sterilized the culture, and the number of CFU dropped below the detection limit. This result is in line with previous observations that treatment with aminoglycosides can lead to very low persister levels or sterilization of bacterial cultures (39, 46, 48).

Aging cultures of various deletion mutants have variable persister formation dynamics. In addition to *hipA7*, several deletion mutants have been reported to have persister levels different from those of the wild type (9, 13, 19, 24, 25, 40). A subset of these was chosen to investigate their persister levels at different stationary-phase durations. Several previous screens (13, 25) have been performed on the Keio collection of strains (1). Therefore, we started with Keio strains (see Fig. S3 in the supplemental material), but it did not escape our attention that the strains might have accumulated second-site compensatory mutations or that the antibiotic resistance cassette might cause polar effects in the operons. Therefore, we reconstructed most of the strains under study in a clean background and removed the resistance gene (Fig. 4). Indeed, slight differences between the Keio collection and the fresh strains were observed.

All of our experiments were performed at a constant concentration of ampicillin. It is possible that the persister levels measured reflect the sensitivities of the strains to the drug. Therefore, we determined the MICs of ampicillin against all the strains under study (see Table S1 in the supplemental material). The changes in antibiotic sensitivity were small and,

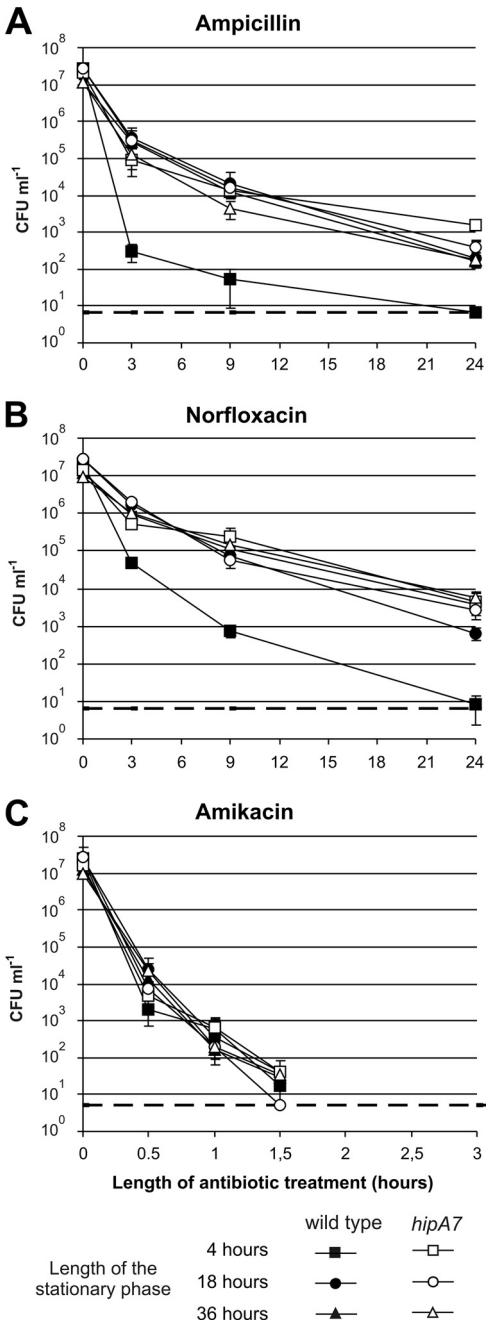

FIG. 3. Dynamics of persister frequency. Stationary-phase cells of MG21 (wild type) and MG22 (*hipA7*) grown in LB filled with GFP were diluted in fresh medium (LB) at the indicated times of the stationary phase. The beginning of the stationary phase was defined as the time when the optical density of the cultures did not increase >5% for 30 min. The persister frequencies of the cultures upon dilution were determined at the indicated incubation times (3, 9, and 24 h) by plating samples on LB plates. The values represent the means of three independent experiments. The error bars indicate the standard errors. The dashed horizontal lines indicate detection limits.

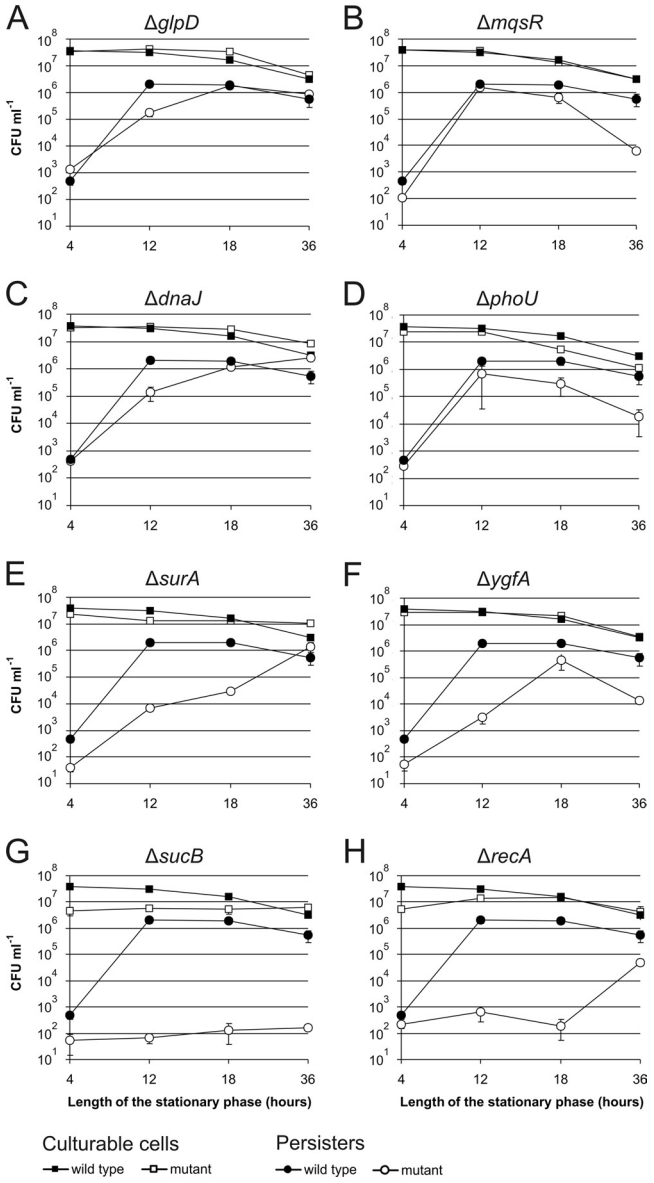

FIG. 4. Frequencies of persisters in single-gene knockout strains. The Δ*sucB* and Δ*recA* strains were from the Keio collection (1); the other strains were freshly constructed from BW25113 (wild type). Stationary-phase cells grown in LB were diluted in ampicillin-containing fresh medium (LB) at the indicated time of the stationary phase. All cultures were inoculated at the same time, and the beginning of the stationary phase was defined as the time when the optical density of the wild-type culture did not increase >5% for 30 min. At the same time points, the number of CFU was determined by plating samples on LB plates. The persister frequencies of the diluted cultures were determined by plating samples on LB plates 3 h after dilution and ampicillin treatment. The values represent the means of at least three independent experiments. The error bars indicate the standard errors.

compared to the more than an order of magnitude higher concentration of the drug used in persister tests, were considered negligible. Therefore, it is expected that all the growing bacteria are rapidly killed independent of the genetic background.

Several types of mutants were identified on the basis of changes in their persister levels (Fig. 4). The *mqsR* (19) and *phoU* (24) knockout strains had persister levels similar to that of the wild type when the inocula were from the beginning of stationary phase, followed by a decrease of more than an order of magnitude at later time points (Fig. 4B and D).

glpD (40), *surA* (13), and *dnaJ* (13) knockout strains had lower levels of persisters than the wild type when the inocula were taken from early-stationary-phase cultures, although the effects (Fig. 4A, C, and E) were smaller than those of the other knockout strains. After 18 h in the stationary phase, these mutants had persister numbers that were comparable to those of the wild type, and after 36 h, they may have exceeded the wild-type levels.

The *sucB* (25) and *ygfA* (13) knockout strains produced lower levels of persisters at every time point examined (Fig. 4F and G), with *sucB* deletion causing considerably larger effects. As we were unable to construct a *sucB* deletion in a clean background, we tested if the effect of the knockout can be compensated for by a plasmid-borne copy of the gene. Indeed, *sucB* expression from the plasmid increased persister frequency, although the wild-type levels were not reached (see Fig. S4 in the supplemental material). As the gene regulation pattern of the chromosomal copy is very probably not reproduced by the plasmid, the partial complementation is not unexpected. The number of persisters also remained low in the case of the *recA* knockout strain (9), increasing only at the last time point examined (Fig. 4H). It is important to note that the *recA* knockout led to large growth defects (data not shown), and therefore, its relevance in the current context is questionable.

The *sucB* strain with the strongest effect on persister levels was examined further. The decrease in persister levels was observed upon 3-h ampicillin treatment (Fig. 4G). To investigate if this holds true for longer ampicillin treatments, both Δ*sucB* and wild-type strains were subjected to prolonged killing by ampicillin. The survival of individual cells that were inoculated into fresh LB medium after 4 h, 18 h, and 36 h in the stationary phase was measured. Like the wild type, Δ*sucB* cells were rapidly killed by ampicillin if the cultures were started with young inocula (Fig. 5B). The wild type became more tolerant of ampicillin treatment as the inoculum became older, but this was not the case for the Δ*sucB* mutant: bacteria died quickly even if taken from the 36-h stationary-phase culture. The growth resumption was estimated by counting nongrowing bacteria using flow cytometry (Fig. 5A). The wild-type strain demonstrated growth resumption dynamics consistent with the data in Fig. 1 and 2, with the aged inocula producing cultures with slower growth resumption. In contrast, the growth resumption of Δ*sucB* cells was rapid regardless of the length of the stationary phase. It is interesting that the *hipA7* strain had increased persister levels without a change in growth resumption kinetics (Fig. 1). This contrasts with the Δ*sucB* strain, where the decreased persister level was paralleled by a generally increased growth resumption speed (Fig. 5).

sucB codes for an enzyme involved in the tricarboxylic acid cycle. Several other enzymes of the same pathway have been previously implicated in modulating antibiotic action (20). Therefore, we tested the effects of *icdA*, *mdh*, and *acnB* deletions on the persister levels (Fig. 6). Indeed, the *acnB* deletion led to considerably decreased persister levels, similar to the

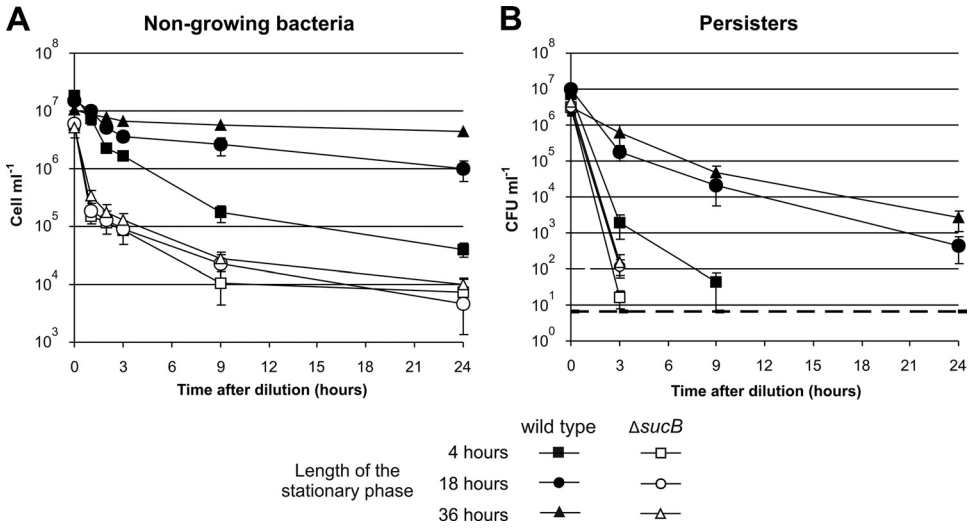

FIG. 5. Dynamics of growth resumption (A) and persister frequency (B) in wild-type (BW25113) and Δ*sucB* strains (from the Keio collection [1]). Stationary-phase cells, transformed with plasmid pETgfp-mut2-AGGAGG(3)-CAT, grown in LB, and containing GFP, were diluted in ampicillin-containing fresh medium (LB) at the indicated points in the stationary phase. The beginning of the stationary phase was defined as the time when the optical density did not increase >5% for 30 min (the criterion was applied separately to the two cultures). (A) The numbers of nondividing cells were measured using flow cytometry by counting cells retaining high GFP content. (B) The persister frequencies of the diluted cultures were determined using ampicillin treatment (lengths are indicated on the *x* axis) and by plating samples on LB plates. The values represent the means of three independent experiments. The error bars indicate the standard errors. The dashed horizontal line in panel B indicates the detection limit.

effect of *sucB*. This is opposed to the other two deletions, which in the first time point caused increased persister levels that merged with the wild-type levels later. Thus, we demonstrated that modulating central metabolism can lead to changed persister levels, although explaining the effects of particular genes requires more thorough experimentation and modeling (14).

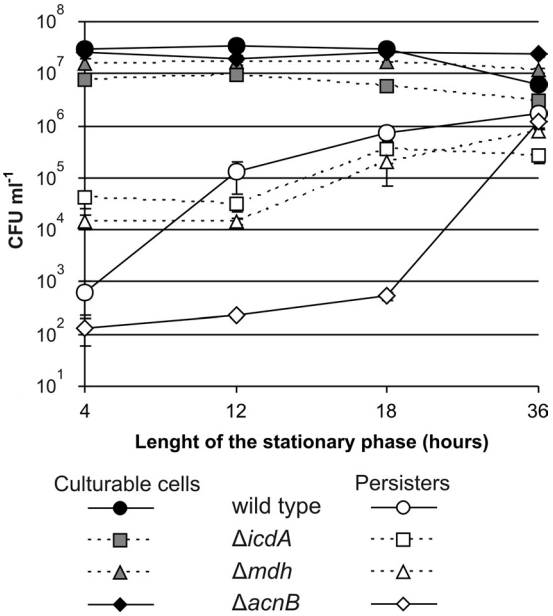

FIG. 6. Frequencies of persisters in strains lacking genes for enzymes involved in the tricarboxylic acid cycle. The strains were tested as described in the legend to Fig. 4.

DISCUSSION

Environmental and genetic factors contribute to the frequency of persisters, and several mutants with altered frequencies have been identified (9, 10, 13, 19, 24, 25, 27, 35, 40). Here, the effect of inoculum age on the persister frequencies of these mutants is described. The difference in the frequencies of persisters between wild-type and mutant strains can be highly dependent on the age of the inoculum. Such a strong dependence on exact experimental conditions might explain why the lists of genes influencing persister formation seldom overlap in different screens. Indeed, most of the strains tested exhibited persister levels different from those of the wild type at only some stationary-phase time points and not others (Fig. 1, 4, 6).

The relative nature of persisters has been noticed before (24–26, 47). The decreased number of persisters in a *phoU* deletion mutant was evident only under certain conditions (24), and results with *sucB* and *ubiF* deletion strains prompted the authors to propose a concept of "deep" and "shallow" persisters (25). Our data are compatible with the concept of the relative nature of persisters.

High sensitivity to the time that inoculated bacteria have spent in the stationary phase can lead to the identification of strains with altered growth rates as persister mutants. In contrast to other knockout strains tested, the *recA* deletion mutant grew significantly more slowly than the wild type. It took 9 h for wild-type *E. coli* to reach the stationary phase in LB, but the *recA* knockout strain reached a similar phase after 24 h (data not shown). Therefore, the cells from these two strains are likely to be in different physiological states, and the different frequencies of persisters might reflect the differences in growth speed and not necessarily the inherent difference in growth resumption. It is important to note that the original *hip* screen

considered only mutants with unchanged growth parameters (27). These criteria have been more relaxed in some of the later screens (24), and therefore, more careful characterization of strains is required.

The levels of persisters can be changed via two mechanisms. In the *sucB* deletion strain, the growth resumption speed of the bulk culture is increased, leading to a decreased persister level. This change, affecting all cells, also influences a small subpopulation of persisters. In contrast, the *hipA7* mutation does not affect the growth parameters of the bulk culture but has a specific effect on a minor subpopulation of cells. Delayed growth resumption among the cells in this subpopulation increases the frequency of persisters. These results suggest one way in which mutants with altered persister frequencies could be categorized.

The results obtained using three different antibiotics indicate that persisters in *E. coli* are not universally tolerant of all agents. Ampicillin, targeting cell wall synthesis, and norfloxacin, targeting topoisomerases, leave a detectable fraction of persisters after 24-h treatment. On the other hand, amikacin, targeting protein synthesis, kills all cells very quickly and effectively sterilizes the culture within 3 h. This points to the interesting possibility that persisters have the capability to synthesize protein long before cell growth and chromosome replication take place; amikacin treatment for 3 h eliminates cells that can withstand 24-h exposure to ampicillin or norfloxacin. This result is consistent with the work of Gefen and coworkers, which described a short period of protein synthesis activity in cells that became dormant again and exhibited delayed growth resumption (12). It has to be noted that some of the previous studies have used a different persister test, treating the stationary-phase cultures with antibiotics followed directly by plating (13, 18, 24, 39). The effect of amikacin in this alternative experimental setup remains to be seen.

E. coli cells are known to accumulate oxidative damage during the stationary phase and may require additional time in fresh media to repair their constituents before resuming growth (8). The results from the *sucB* deletion strain suggest that it is possible to avoid such a delay—it maintains quick recovery and cell viability longer than the wild type. The fact that the wild type has not acquired *sucB*-like properties suggests the possibility that delayed growth resumption is favored during evolution.

Persistence could be a bet-hedging strategy, generating phenotypic heterogeneity under uncertain conditions (3). It is possible that expectations for stable favorable conditions change during the time spent in the stationary phase. More persisters (delayed growth resumption) after a longer stationary phase could reflect a higher expectation of catastrophic events occurring during the next growth phase.

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